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Rapid Screening for Targeted Genetic Variants via High-Resolution Melting Curve Analysis

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Abstract

Background—Current methods for the detection of single nucleotide polymorphisms (SNPs) associated with aberrant drug-metabolizing enzyme function are hindered by long turnaround times and specialized techniques and instrumentation. In this study, we describe the development and validation of a high-resolution melting (HRM) curve assay for the rapid screening of variant genotypes for targeted genetic polymorphisms in the cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP3A5.

Methods—Sequence-specific primers were custom-designed to flank nine SNPs within the genetic regions of aforementioned drug metabolizing enzymes. PCR amplification was performed followed by amplicon denaturation by precise temperature ramping in order to distinguish genotypes by melting temperature (T_m). A standardized software algorithm was used to assign amplicons as ‘reference’ or ‘variant’ as compared to duplicate reference sequence DNA controls for each SNP.

Results—Intra-assay (n=5) precision of T_m s for all SNPs was 0.19%, while inter-assay (n=20) precision ranged from 0.04% to 0.21%. When compared to a reference method of Sanger sequencing, the HRM assay produced no false negative results, and overcall frequency ranged from 0–26%, depending on the SNP. Furthermore, HRM genotyping displayed accuracy over input DNA concentrations ranging from 10–200 ng/ μ L.

Conclusions—The presented assay provides a rapid method for the screening for genetic variants in targeted CYP450 regions with a result of ‘reference’ or ‘variant’ available within 2 h from receipt of extracted DNA. The method can serve as a screening approach to rapidly identify individuals with variant sequences who should be further investigated by reflexed confirmatory testing for aberrant cytochrome P450 enzymatic activity. Rapid knowledge of variant status may aid in the avoidance of adverse clinical events by allowing for dosing of normal metabolizer patients immediately while identifying the need to wait for confirmatory testing in those patients who are likely to possess pharmacogenetically-relevant variants.

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Keywords

High resolution melting; single nucleotide polymorphisms; CYP450

Introduction

Targeted genotyping of polymorphic regions for pharmacogenomics applications is conducted by a variety of molecular techniques. Typically, molecular methods for genetic analysis begin with PCR amplification of the targeted region; post-amplification, genetic products are detected via methods including hybridization, primer extension, ligation, or invasive cleavage [1]. Such methods may aid in indicating disease prognosis and treatment responses, and they are also relevant to the identification of variations associated with inherited disorders. While a number of methodologies are available for genotyping of polymorphisms, common approaches such as direct sequencing and microarrays are limited by long turnaround times or the need for specialized instrumentation. An important consideration in the selection of a molecular platform for targeted genetic testing is the identification of a technique that can meet the needs of an increasingly automated clinical laboratory to accommodate the rapid delivery of genetic information in order to provide clinical decision support.

High-resolution melting (HRM) curve analysis is a simple, rapid, and cost-effective method for genotyping that employs differences in the melting temperature (T_m) of targeted amplicons to identify genetic variations [2, 3]. With HRM, PCR-amplified DNA is incrementally heated to denature double-stranded DNA (dsDNA) into single-stranded nucleotide sequences, thereby resulting in a sharp decrease in dsDNA detection via an intercalating nucleic acid dye. Various strengths of H-bonds, dependent upon specific nucleotide base composition of the target genetic sequence, result in unique melting curve profiles for a given amplicon. Therefore, genetic variants are identified by differential DNA T_m s and melting curve profiles when compared to reference genomic sequences. Notably, because the melting step may be conducted at the completion of amplification on the same thermal cycler instrument and within the same run, opportunities for processing errors and contamination are minimized. HRM approaches have been previously applied for the detection of both inherited and somatic mutations [4–8].

The cytochrome P450 (CYP450) superfamily of enzymes is responsible for the metabolism of over 75 percent of commonly prescribed therapeutic agents [9, 10]. Single nucleotide polymorphisms (SNPs) in the genes encoding these enzymes may significantly impact downstream enzymatic activity, resulting in increased variability to drug response. Advancement of genomic technologies has led to increased knowledge of gene-drug relationships, and expanding references are available to correlate genotype to drug-metabolizing phenotype as well as to guide drug dosing [11, 12]. Further, knowledge of pertinent CYP450 variations may aid in selection of both drug and dose for maximum efficacy and/or avoidance of adverse drug reactions [13]. Conventional genotyping techniques typically utilized for CYP450 SNP detection are associated with long turnaround times that may cause delays in therapy or premature dosing without knowledge of phenotype

status, thereby increasing risk for adverse drug reactions [14]. Thus, a rapid, targeted genotyping approach may aid significantly in minimizing drug-related adverse events and improving therapeutic outcomes by providing a same-day rule out of normal metabolizer patients [15, 16].

In this study, we describe the development and validation of a method for the rapid screening for variant genotypes for targeted genetic polymorphisms of the CYP2C9, CYP2C19, and CYP3A5 enzymes via HRM analysis. The described validation serves as a proof-of-concept for the assessment of the analytical metrics of a rapid SNP genotyping assay. While commercial HRM platforms are currently available for SNP genotyping, the presented assay utilizes a conventional thermal cycler instrument and provides an expandable, open-access model to allow for the detection of any variations of interest. The particular alleles detected in this study (*CYP2C9**2, *CYP2C9**3, *CYP2C19**2, *CYP2C19**3, *CYP2C19**17, *CYP3A5**2, *CYP3A5**3, *CYP3A5**6, *CYP3A5**7) are included due to their significant impact on enzymatic activity and drug metabolism [17–22]. The presented HRM assay can rapidly identify individuals with variant sequences who should be further assessed for aberrant CYP450 enzymatic activity. The detected polymorphisms may exhibit relatively low abundance within the general population but are characterized by high clinical utility. Thus, such a screening approach would likely serve to rule out the normal metabolizer majority from further testing while identifying those who may be at risk for adverse drug reactions.

Materials and methods

Primer design

Custom sequence-specific primers were designed to amplify regions flanking targeted SNPs for *CYP2C9* (*2, *3), *CYP2C19* (*2, *3, *17), and *CYP3A5* (*2, *3, *6, *7) (Supplemental Table 1) and were acquired from Integrated DNA Technologies (IDT, Coralville, IA, USA). Primer T_m s and design suitability parameters were assessed via the IDT OligoAnalyzer 3.1 (<https://www.idtdna.com/calc/analyser>), while specificity was confirmed using the NCBI BLAST tool for sequence homology (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). NCBI dbSNP (<http://www.ncbi.nlm.nih.gov/SNP>) was utilized to confirm that amplicons did not contain any other identified rare variants other than the SNPs of interest.

DNA templates

Reference sequence, heterozygous, and homozygous control template DNA samples containing each SNP of interest were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA) after obtaining proper institutional approval. Genotypes of control DNA samples were confirmed using the Coriell repository of verified pharmacogenetic variants (<https://catalog.coriell.org/0/Sections/BrowseCatalog/Pharmacogenetics.aspx>) and the NCBI 1000 Genomes Browser (<http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes>). Genotypes were also independently verified by Sanger Sequencing using the aforementioned sequence-specific primers (**data not shown**).

For concordance studies, de-identified remnant human whole blood specimens were obtained from the Johns Hopkins Hospital Core Laboratories. Specimen acquisition was conducted with Institutional Review Board (IRB) approval from the Johns Hopkins University School of Medicine. DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Immediately following extraction, DNA concentrations and purity were measured with a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), and the extracted samples were subsequently aliquotted and stored frozen at -20°C . DNA samples with concentrations below $10\text{ ng}/\mu\text{L}$ were not included in concordance studies.

PCR and high resolution melting (HRM) reagents and procedure

PCR followed by HRM was performed using a Rotor-Gene Q 5plex HRM Platform thermal cycler instrument (QIAGEN). For a single reaction, the following materials were combined inside a sterile PCR workstation that was pre-treated with UV light for 10 minutes: $1\text{ }\mu\text{L}$ of $100\text{ ng}/\mu\text{L}$ control DNA template, 400 nM each of forward and reverse primers, $12.5\text{ }\mu\text{L}$ of Type-It HRM PCR Kit containing EvaGreen dsDNA-binding fluorescent dye (QIAGEN), and $9.5\text{ }\mu\text{L}$ of PCR-grade water (QIAGEN). Analytical runs that included concordance samples were run with $100\text{ ng}/\mu\text{L}$ each of reference, heterozygous, and homozygous DNA controls in duplicate, both at the beginning and end of the run. PCR cycling profile and HRM temperature ramp conditions for each SNP are detailed in Supplemental Table 2. Rotor-Gene Q software v. 2.0.3 (QIAGEN) generated melting profile curves by quantifying detected fluorescence at each HRM temperature increment. Post-amplification and melting, PCR cycling graphs and HRM curves were manually reviewed to ensure proper amplification and melting; derivative fluorescence-versus-temperature (dF/dT) plots were assessed to verify one peak per sample, indicating that a single, specific product was amplified and melted. Additionally, PCR curves were evaluated to ensure the absence of amplification within no-template control (NTC) samples.

Post-amplification and melting, reference sequence control profiles were genotype-assigned using the Rotor-Gene Q software. All other curves were normalized to the fluorescence values of the designated reference samples, thereby generating HRM difference plots. Subsequently, by employing a user-defined genotype confidence threshold, melting curve profiles were assigned a genotype relative to the run-specific assigned reference melting profile. Unknown samples with melting profiles (relative to the reference-assigned control) above the user-defined threshold were designated as 'reference' and samples below the threshold were designated as 'variant.' The run-specific genotype confidence threshold was defined as the overall average of normalized, extracted melting curve profile results for the duplicate reference and duplicate heterozygous controls $[(\text{reference A} + \text{heterozygous A} + \text{reference B} + \text{heterozygous B})/4]$ included in an analytical run, rounded to the nearest whole number percentage (Supplemental Figure 1). For the *2C19*2* allele only, this average was further multiplied by 1.3 for the final threshold value; this was optimized to the PCR-cycling conditions specific for this allele. Using this algorithm, both heterozygous and homozygous samples were collectively designated as 'variant,' facilitating a screening approach to differentiate reference from variant samples. As a suitability check for reagents and instrumentation, the duplicate heterozygous and homozygous controls included at the

beginning and end of the run were required to be correctly called as ‘variant’ by the software in order for the run to be considered viable for unknown sample genotype auto-calling.

Assay validation

Performance of the HRM screening assay was assessed by validation of primer selectivity, assessment of precision of resultant T_m s, assessment of effect of input DNA concentration on genotype calling, and concordance of the HRM genotyping to Sanger sequencing.

Selectivity—Primer selectivity was verified by agarose gel electrophoresis and Sanger sequencing. Template control DNA was PCR-amplified using the custom-designed primers described above and then loaded into a 1.5% agarose gel containing ethidium bromide to visualize product bands. Amplified products were also submitted to the Johns Hopkins Hospital Genetic Core Research Facility for Sanger sequencing. Results were aligned to desired reference sequences using NCBI GRCh38 human genome assembly notation with Sequencher sequence analysis software (Gene Codes, Ann Arbor, MI, USA).

Intra-run and inter-run precision of melting temperatures—The intra-assay precision of HRM resultant T_m s was assessed using $n=5$ replicates of each DNA input control genotype (reference, heterozygous, homozygous) per SNP within the same run. The inter-assay precision of T_m was assessed using duplicate samples of each control genotype over ten separate runs on ten days for a total of $n=20$ replicates per genotype per SNP. Imprecision was calculated using the coefficients of variation (%CVs) from resultant T_m s for control DNA samples.

DNA input studies—For each SNP, DNA samples of each genotype were tested in triplicate at input concentrations of 10, 50, 100, 150, and 200 ng/ μ L to assess precision of resultant HRM T_m s and accuracy of genotype calling at each concentration. Means, standard deviations (SDs), and %CVs of T_m s were compared across the DNA input concentration range (**data not shown**), and HRM genotype calls at each concentration were compared to that of the standard 100 ng/ μ L input concentration.

Concordance analysis—Concordance studies were performed to determine the accuracy of HRM genotype calling; HRM analysis was compared to traditional Sanger sequencing. 45 de-identified human DNA samples of unknown genotypes were analyzed by HRM to obtain a call of ‘reference’ or ‘variant’ for each allelic polymorphism. Subsequently the same samples were analyzed by Sanger sequencing, which was performed by the Johns Hopkins Genetic Resources Core Facility via a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Genotype calls for each sample were compared to determine overall method agreement. A genotype overcall, or false positive, was defined as the HRM assay calling a sample ‘variant’ while the same sample was called ‘reference’ by Sanger sequencing. A genotype undercall, or false negative, was defined as the HRM assay calling a sample ‘reference’ while the same sample was called ‘heterozygous’ or ‘homozygous’ by Sanger sequencing. From counts of true and false positives and negatives, assay sensitivity, specificity, positive predictive value, and negative predictive value was assessed for each SNP (**data not shown**).

Results

The CYP450 SNP alleles included in the rapid HRM screening assay are displayed in Table 1. Of note, the metabolism phenotype depends on the diplotype rather than the individual allele. Sequence-specific primers were custom designed to flank polymorphisms of interest (Supplemental Table 1). All primer oligonucleotides were designed with similar T_m s to best harmonize PCR cycling conditions across all SNPs assessed in the study. Amplicon lengths ranged from 150 to 208 base pairs (Supplemental Table 1). Primer pairs were evaluated for genomic region specificity by agarose gel electrophoresis, which confirmed single product amplification at the expected base pair length for each SNP (Supplemental Figure 2). Product amplicons were also verified via Sanger sequencing (**data not shown**).

Upon verification of primer specificity, PCR conditions were optimized for amplicon production and HRM denaturation and analysis. Reference, heterozygous, and homozygous control DNA templates were PCR-amplified, and amplified material was subjected to denaturation during melting curve analysis. Two primary amplification and melting protocols were developed, one for *CYP3A5* (*2, *3, *6, *7), and one for both *CYP2C9* (*2, *3) and *CYP2C19* (*3, *17). Additionally, due to the decreased G-C content flanking the *CYP2C19**2 polymorphism location, an allele-specific amplification and HRM protocol was utilized, which involved extended amplification cycles and a narrower temperature ramp for melting curve discrimination.

Shifts in T_m and melting profiles relative to reference were observed for the heterozygous and homozygous samples (Figure 1). HRM difference plots illustrated curve data normalized to the designated reference samples; variant sequences displayed relative values ranging from 3 to 14 times that of reference (Figure 2). Curves shifted in the positive or negative T_m direction from reference depending on the characteristic base pair change of each SNP. Guanine or cytosine substitutions increased amplicon T_m (see positive curve shifts from reference of *CYP2C9**3 and *CYP3A5**3 in Figure 2), while thymine or adenine substitutions decreased amplicon T_m (see negative curve shifts from reference of *CYP2C9**2, *CYP2C19**2, *CYP2C19**3, *CYP2C19**17, *CYP3A5**2, *CYP3A5**6 in Figure 2). The base pair insertion of an adenine (*CYP3A5**7) also negatively impacted T_m . A homozygous control DNA sample for *CYP3A5**2 was not included in analysis because a sample with both minor alleles has not been identified or reported in studies across several ethnic populations [23, 24].

Precision analysis of the resultant amplicon T_m s yielded %CVs ranging from 0.01–0.19% for intra-run studies and 0.04–0.21% for inter-run studies (Table 2). As blood-extracted DNA may be highly variable in nucleic acid concentration, additional evaluation studies were performed to determine whether DNA input concentration affected the ability of the HRM assay to accurately call genotypes. For each variant of interest, reference, heterozygous, and homozygous control DNA samples were prepared at concentrations ranging from 10 to 200 ng/ μ L and subsequently tested by the same amplification and HRM procedures described above. Variation in input DNA concentration did not cause significant changes in resultant T_m s; %CVs for all concentrations collectively within each SNP ranged from 0.03–0.21% (**data not shown**). Notably, input DNA concentrations from 10–200 ng/ μ L

did not adversely affect genotype calling. Representative HRM curves and genotype results for *CYP3A5**3 using DNA inputs of 10, 50, and 100 ng/μL are shown in Supplemental Figure 3.

Standardized data analysis workflow is illustrated in Figure 3. Upon PCR amplification and product melting, the dF/dT plot was assessed for one peak per sample, indicating that a single, specific product was amplified and melted. Fluorescence data were normalized, and reference controls were user-designated in order to generate difference plots to distinguish variant sequences from reference. The software then compared the melting curve profile of each unknown sample to that of the reference -assigned control using a run-dependent genotype confidence threshold in order to assign genotypes as either 'reference' or 'variant,' as detailed in Materials and Methods.

Concordance studies were performed using 45 human DNA samples of unknown genotype. Each unknown sample was simultaneously analyzed using the HRM assay and a reference Sanger method. Resultant genotype calls from both techniques were compared for each assessed SNP. Figure 4 illustrates a representative concordance analysis for three unknown samples assessed for *CYP3A5**3. Table 3 summarizes the overall agreement of the HRM assay with Sanger sequencing for all SNPs. Overall accuracy ranged from 73–78% for *CYP2C9*, 93–100% for *CYP2C19*, and 98–100% for *CYP3A5*. While overcalling ranged from 10–12 of the 45 samples tested for *CYP2C9*, overcalls were limited to 0–3 of 45 samples for *CYP2C19* and *CYP3A5*. Importantly, no undercalls were observed for any SNP. This translated to an assay sensitivity of 100% as well as a negative predictive value of 100% for each SNP (**data not shown**), indicating the assay's ability to rule out individuals without the targeted variants. Specificity ranged from 69–72% for *CYP2C9*, due to the relatively higher frequency of overcalls for those SNPs, while specificity ranged from 86–100% for all other SNPs.

Discussion

The uptake of pharmacogenetics testing to guide drug dosing has been hindered by a number of factors, including rigorous clinical decision support tools, interfacing capabilities with laboratory and hospital information systems, as well as limited financial reimbursement [16]. Another major reason for the lack of clinician incentive to routinely utilize pharmacogenetics testing is the long turnaround times associated with current genotyping methodologies. The described HRM assay rapidly identifies patients with variant alleles within a single-run, single-instrument workflow on a conventional thermal cycler instrument. The SNPs included for detection by this assay were selected based on their established significant impact on the metabolism of a wide variety of pharmaceuticals. As these polymorphisms may have relatively low abundance in the population but have the potential for significant clinical utility, the described screening approach serves to rule out patients at risk for adverse drug reactions. From receipt of extracted DNA, the time to HRM result of either 'reference' or 'variant' is less than 2 hours. Additionally, the described HRM screening assay is more affordable than other available genotyping techniques. In addition to significant savings in upfront instrument cost, reagent cost per sample is estimated at \$1, compared to approximately \$25 for pyrosequencing and \$6 for Sanger sequencing, as

provided by the Johns Hopkins Molecular Diagnostics Laboratory. Notably, HRM-based platforms are currently commercially available for allelic discrimination. For instance, the Applied Biosystems 7500 Fast (Thermo Scientific) has been marketed for quantitative PCR and HRM analysis with rapid turnaround times. In terms of performance, the described method is comparable to other commercially available HRM-based assays. However, important considerations for such platforms include scalability and system flexibility. Further, allelic discrimination software via HRM may be platform specific and should be carefully evaluated for each genetic polymorphism of interest. The detailed description of software evaluation and HRM threshold determination for multiplexed genotype screening are strengths of the presented work.

As the HRM assay serves as a screening mechanism, priority lies in identifying all potentially positive patients and progressing them to confirmatory testing. While previously described HRM approaches have been used for diagnostic identification of genetic variations, it is important to note that HRM only identifies the general presence of a variant on the target amplicon. It is possible that other rare adjacent SNPs could alter the amplicon T_m and initiate a 'variant' result even though they are not the aberration intended to be detected. Thus, confirmatory testing is required to identify the true variation causing the T_m shift for therapy-guiding purposes. Furthermore, a limitation of the HRM approach is the possible inability to distinguish heterozygous and homozygous samples (Figures 1–2). However, the described assay is purposefully designed to collectively call heterozygotes and homozygous as 'variant' such that these samples would reflex to confirmatory testing for more specific genotype calling. We envision that this screen-reflex approach would be similar to the approach that is currently standard for drugs of abuse screening, in which positive results from broad-spectrum immunoassays are confirmed by more specific methodologies such as mass spectrometry [25].

The genotype confidence threshold within our HRM analysis, used to call the unknown samples as either 'reference' or 'variant,' is set using values from controls within the same run to account for inter-run variability; its calculation is designed conservatively to err on the side of overcalling rather than undercalling variants. In our concordance studies, no false-negative results were identified. One limitation to the described method is the frequency of overcalls (22–27%) for the *CYP2C9* (*2 and *3) alleles. During assay development, several pairs of primers and PCR amplification conditions were evaluated; however, all other primer pairs and PCR conditions were inferior to the methods described in the manuscript (data not shown). Minimizing the detection of false negative results (as indicated by no overcalls for all evaluated alleles) was preferable as any overcalls would be reflexed using an orthogonal, confirmatory method. Further, because the allelic frequency of many of the variants is relatively low in the population (Supplemental Table 3), we predict that the screening approach would greatly minimize the need for confirmatory sequencing for all patients and would serve to rule out a significant number of patients who are not at risk for adverse drug reactions related to the selected CYP450 variations. Furthermore, the cost savings from using a more expensive confirmatory methodology only for a small percentage of samples, those called variant by the HRM screen, rather than using that methodology for all samples upfront would be largely significant.

Constraints of high sequence homology amongst the CYP450 regions ultimately led to three different amplification and melting protocols to encompass all nine alleles identified by the assay. While not all SNPs could be tested within the same run, the thermal cycler software allows for pre-programming of all cycling and melting conditions to maintain various protocols used within the assays. Some SNPs, such as those of the CYP3A5 family (*CYP3A5* *2, *3, *6, and *7) could be multiplexed together within the same run due to their identical reaction conditions. An additional constraint of the presented approach is that the HRM assay can only identify as ‘variant’ those patients containing the alleles for which primers were designed within each of the three genes. Other mutant alleles have been reported for *CYP2C9*, *CYP2C19*, and *CYP3A5*. However, the presented assay is designed to detect the most frequent and well-studied alleles of these genes [26–28]. The minute remainder of possible aberrations within these genes, which may also impact enzymatic activity, would not be identified by this assay. This limitation should be taken into consideration as results are interpreted. The presented nine-SNP HRM panel is described as proof-of-concept, and the open-access nature of the described lab-developed technology allows for expansion via the design of primers to detect other alleles or variations of interest.

The HRM screening assay may be implemented into the routine clinical laboratory in combination with reporting mechanisms in order to offer personalized, guided therapy for patients [11, 12]. Patients identified as ‘reference’ in the setting of a particular relevant drug would be considered of the ‘normal metabolizer’ phenotype and may be dosed immediately upon receipt of the result. However, patients identified as ‘variant’ should be subjected to further confirmatory testing to identify a specific variant(s) in order to classify them as of the ‘poor,’ ‘intermediate,’ or ‘rapid metabolizer’ phenotypes in advance of drug administration. Confirmatory testing may be performed via such techniques as fluorescently-labeled allele-specific probes or direct sequencing. The genotyping results may complement other technologies in the clinical laboratory, such as drug level quantification by therapeutic drug monitoring, in order to monitor for safe and effective drug metabolism. The initial result of ‘reference’ or ‘variant’ could be available on the same day of the visit. Blood collection and DNA extraction steps may add an estimated 0.5–1 h to the 2 h HRM turnaround time. However, utilizing other collection methods such as buccal swabs or saliva may expedite this process. Such non-invasive collection techniques have been explored successfully with the described assay, though K2-EDTA blood samples were used in the validation presented here. Notably, other blood preservatives, such as heparin, may interfere with PCR assays [29]; however, alternative preservatives were not evaluated in this study.

Barriers to implementation of real-time pharmacogenetics in the clinic include the need for guidance in which specific genes for which to consider testing and how to interpret results in light of the specific drug(s) being prescribed to the patient. With the aid of resources such as the guidelines from the Clinical Pharmacogenetics Implementation Consortium (CPIC) [11], the Dutch Pharmacokinetics Working Group (DPWG) [30,31], and other similarly-focused organizations across the globe, an electronic prompt may trigger the ordering of particular genotypes once a particular drug known to have pharmacogenetic implications is prescribed. For instance, the initial selection of the drug tacrolimus by a provider may prompt an order for a CYP3A5 genotype [32]. With the presented assay, the relevant alleles *CYP3A5* *2, *3, *6, and *7 could be indicated as a multiplexed panel, or be ordered a la carte. Genotype

results with interpretation guidance would be provided for dosing of the particular drug initially ordered, according to CPIC, DPWG, and other nationally-recognized guidelines. Such preemptive approaches have been recently implemented within a few institutions [33], though results are typically not currently available in real-time. The described assay is currently being used in downstream studies to assess its potential clinical utility.

Conclusions

In summary, the presented assay offers rapid identification of patients with variant sequences in genetic regions coding for pharmacogenetically-relevant CYP450 enzymes. This method can be used to screen and rule out samples prior to targeted, confirmatory testing in order to distinguish in real-time patients who should receive alternative therapeutic regimens due to risk of decreased drug efficacy or adverse drug reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HRM	high-resolution melting
T_m	melting temperature
dsDNA	double-stranded DNA
CYP450	cytochrome P450
SNP	single nucleotide polymorphisms
IRB	Institutional Review Board
NTC	no-template control

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Figure 1.

HRM curves distinguish genotypes. For each SNP, representative HRM curves of reference (black curves), heterozygous (green curves), and homozygous (red curves) DNA are illustrated. Single nucleotide base pair mutations caused a shift in melting temperature of the target amplicon, allowing for visual separation of genotypes with HRM curves. No homozygous control DNA template was available for CYP3A5*2 (F).

Figure 2.

HRM difference plots. Differences in melting curve shape were visualized by subtracting the curves from a reference sequence control curve. Normalized fluorescence minus reference (y-axis) was plotted against temperature (x-axis) for each SNP. Heterozygous (green curves) and homozygous (red curves) samples were clearly delineated from reference samples (black curves). With the described HRM screening approach, both heterozygous and homozygous samples would be collectively called as ‘variant.’

Figure 3.

HRM analysis workflow for genotype calling. After amplification and melting (A, B), a derivative fluorescence-versus-temperature plot was assessed for a single peak at the melting temperature (C). Fluorescence was normalized (D), and a fluorescence-minus-reference plot delineated genotypes (E). Software called genotypes as reference or variant based on a user-defined genotype confidence threshold (F). The software's terminology of 'wild type' is equivalent to our use of 'reference.'

Figure 4.

Concordance with Sanger sequencing. A representative example is shown for CYP3A5*3, a substitution of thymine for cytosine. With Sanger sequencing, reference samples displayed thymine at the SNP location (A), homozygous samples displayed cytosine (C), and heterozygous samples displayed peaks for both alleles (B). HRM difference plots show the same unknown samples (cyan curves) alongside DNA controls of known genotype (D–F).

Table 1

SNPs detected in the rapid CYP450 HRM screening assay

Gene	Allele	Reference SNP cluster ID	Polymorphism ^a	Enzyme Activity	Metabolism Phenotype
CYP2C9	*2	rs1799853	C>T	Nonfunctional	Poor/Intermediate
	*3	rs1057910	A>C	Nonfunctional	Poor/Intermediate
CYP2C19	*2	rs4244285	G>A	Nonfunctional	Poor/Intermediate
	*3	rs4986893	G>A	Nonfunctional	Poor/Intermediate
	*17	rs12248560	C>T	Increased	Ultra-rapid
CYP3A5	*2	rs28365083	G>T	Nonfunctional	Poor/Intermediate
	*3	rs776746	T>C	Nonfunctional	Poor/Intermediate
	*6	rs10264272	C>T	Nonfunctional	Poor/Intermediate
	*7	rs41303343	insA	Nonfunctional	Poor/Intermediate

^aNCBI reference sequence notation GRCh38.p2 NC_000010.11 (2C9, 2C19) and NC_000007.14 (3A5).

Table 2

Precision and reproducibility assessment of melting temperature results

		Intra-assay precision (n=5)			Inter-assay precision (n=20)		
		Mean T _m (°C)	SD (°C)	%CV (%)	Mean T _m (°C)	SD (°C)	%CV (%)
<i>CYP2C9</i> *2	Reference	83.77	0.02	0.02	83.92	0.06	0.07
	Heterozygous	83.63	0.02	0.03	83.83	0.04	0.05
	Homozygous	83.48	0.01	0.01	83.76	0.05	0.05
<i>CYP2C9</i> *3	Reference	83.49	0.02	0.02	83.89	0.07	0.08
	Heterozygous	83.76	0.05	0.06	84.16	0.06	0.08
	Homozygous	84.00	0.02	0.02	84.48	0.06	0.08
<i>CYP2C19</i> *2	Reference	74.83	0.01	0.02	74.80	0.04	0.06
	Heterozygous	74.64	0.03	0.04	74.68	0.06	0.08
	Homozygous	74.55	0.01	0.02	74.61	0.04	0.06
<i>CYP2C19</i> *3	Reference	78.22	0.03	0.03	78.47	0.11	0.14
	Heterozygous	78.01	0.04	0.05	78.30	0.10	0.12
	Homozygous	77.97	0.01	0.02	78.25	0.09	0.11
<i>CYP2C19</i> *17	Reference	76.01	0.06	0.08	76.24	0.16	0.21
	Heterozygous	75.80	0.06	0.08	75.98	0.09	0.12
	Homozygous	75.79	0.07	0.09	75.97	0.11	0.14
<i>CYP3A5</i> *2	Reference	81.17	0.02	0.02	81.02	0.07	0.09
	Heterozygous	81.10	0.02	0.02	80.83	0.07	0.09
	Reference	80.76	0.04	0.05	80.66	0.04	0.05
<i>CYP3A5</i> *3	Heterozygous	80.98	0.09	0.11	80.70	0.03	0.04
	Homozygous	81.23	0.07	0.08	80.73	0.04	0.05
	Reference	75.99	0.06	0.07	75.91	0.06	0.08
<i>CYP3A5</i> *6	Heterozygous	76.12	0.04	0.06	75.93	0.08	0.11
	Homozygous	76.10	0.14	0.19	75.85	0.08	0.11
	Reference	80.11	0.05	0.06	80.52	0.11	0.13
<i>CYP3A5</i> *7	Heterozygous	80.30	0.01	0.02	80.61	0.10	0.12

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	Intra-assay precision (n=5)			Inter-assay precision (n=20)		
	Mean T _m (°C)	SD (°C)	%CV (%)	Mean T _m (°C)	SD (°C)	%CV (%)
	80.42	0.05	0.06	80.54	0.07	0.09
	Homozygous					

Table 3

Concordance of the CYP450 HRM screening assay with Sanger sequencing

	HRM		Sanger			Overall Frequency	Undercall Frequency	Overall Agreement
	Refer- ence	Variant	Refer- ence	Hetero- zygous	Homo- zygous			
CYP2C9*2	26	19	36	9	0	10/45	0/45	78%
CYP2C9*3	27	18	39	5	1	12/45	0/45	73%
CYP2C19*2	31	14	31	13	1	0/45	0/45	100%
CYP2C19*3	41	4	44	1	0	3/45	0/45	93%
CYP2C19*17	41	4	44	1	0	3/45	0/45	93%
CYP3A5*2	43	2	43	2	0	0/45	0/45	100%
CYP3A5*3	6	39	7	8	30	1/45	0/45	98%
CYP3A5*6	44	1	44	0	1	0/45	0/45	100%
CYP3A5*7	42	3	43	2	0	1/45	0/45	98%